SOME OBSERVATIONS ON CHLAMYDOMONAS MICROHALOPHILA SP. NOV.[1]

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During the summer of 1958 the writer became interested in the profuse blooms of a species of *Chlamydomonas* in some barrels of water stored at the head of the Supply Department dock, Marine Biological Laboratory, Woods Hole, Massachusetts.[2] Previous morphological and cytological work on various species of *Chlamydomonas*, especially that of Bold (1949), and Buffaloe (1958), the second of which summarized discrepancies in chromosome numbers in several species, impelled the writer to investigate this cytologically favorable organism.

Materials and Methods

Samples of water containing the organism were inoculated into sterile soil-water tubes (Pringsheim, 1946) and tubes containing an inorganic solution (Bold, 1949) fortified with 5% supernatant from soil-water medium.

Twenty clonal cultures were started by introducing single cells in sterile soil-water tubes. In the soil-water tubes and inorganic salt medium with soil-water supernatant, the growth of the organism was never as good as that observed in the natural habitat. Only a very delicate green phototactic ring appeared at the surface of the culture solutions. A sample of water from the barrel in which the organisms occurred was filtered several times and analyzed for salt content. The latter was found to be approximately 0.022 N in NaCl after titrating for the Cl^- ion by the Mohr method. Sea water from the coast of Texas taken from the Port Aransas area proved to be approximately 0.66 N in NaCl. To obtain the same number of moles of NaCl for a culture solution, as present in the original habitat, 33 ml. of the Gulf sea water were diluted to one liter by adding inorganic medium and soil-water supernatant. After considerable experimentation excellent growth was obtained in a medium of the following composition:

inorganic medium (Bold, 1949)	917 ml.
soil-water supernatant	50 ml.
Gulf sea water	33 ml.

This medium supported good growth both in the liquid state and when solidified with agar.

The addition of sea water to the medium, although not essential for growth,

[1] Investigation initiated while the author was Assistant in the Marine Botany Course, summer of 1958. The author wishes to acknowledge gratefully the friendly help and suggestions given by Dr. Harold C. Bold in the development of this paper.

[2] The same organism was present in these barrels as long ago as 1948 and seems to recur every year.

proved very stimulatory to the algal cultures. It was also of interest to note that six of the more vigorous clones would grow in media with much higher concentrations of sea water.

With this evidence that the organism grows best in relatively high concentrations of salts, as compared with that in the more commonly employed algal culture media, a modified Knop's solution was compounded as follows:

10% $Ca(NO_3)_2$	10 ml.	Gulf sea water (approx. 4.3% salts by weight)	33 ml.
5% KNO_3	5 ml.	Sterile rain water	892 ml.
5% $MgSO_4 \cdot 7H_2O$	5 ml.	Soil-water supernatant	50 ml.
5% KH_2PO_4	5 ml.		

This solution has a salt concentration more than five times that of Bold's (1949) inorganic medium. This solution, with the sea water substituted for 33 ml. of sterile water, contains approximately 0.32% salts by weight, and was employed as the culture medium throughout the remainder of the observations. One clonal culture was inoculated into four sets of tubes containing the modified Knop's

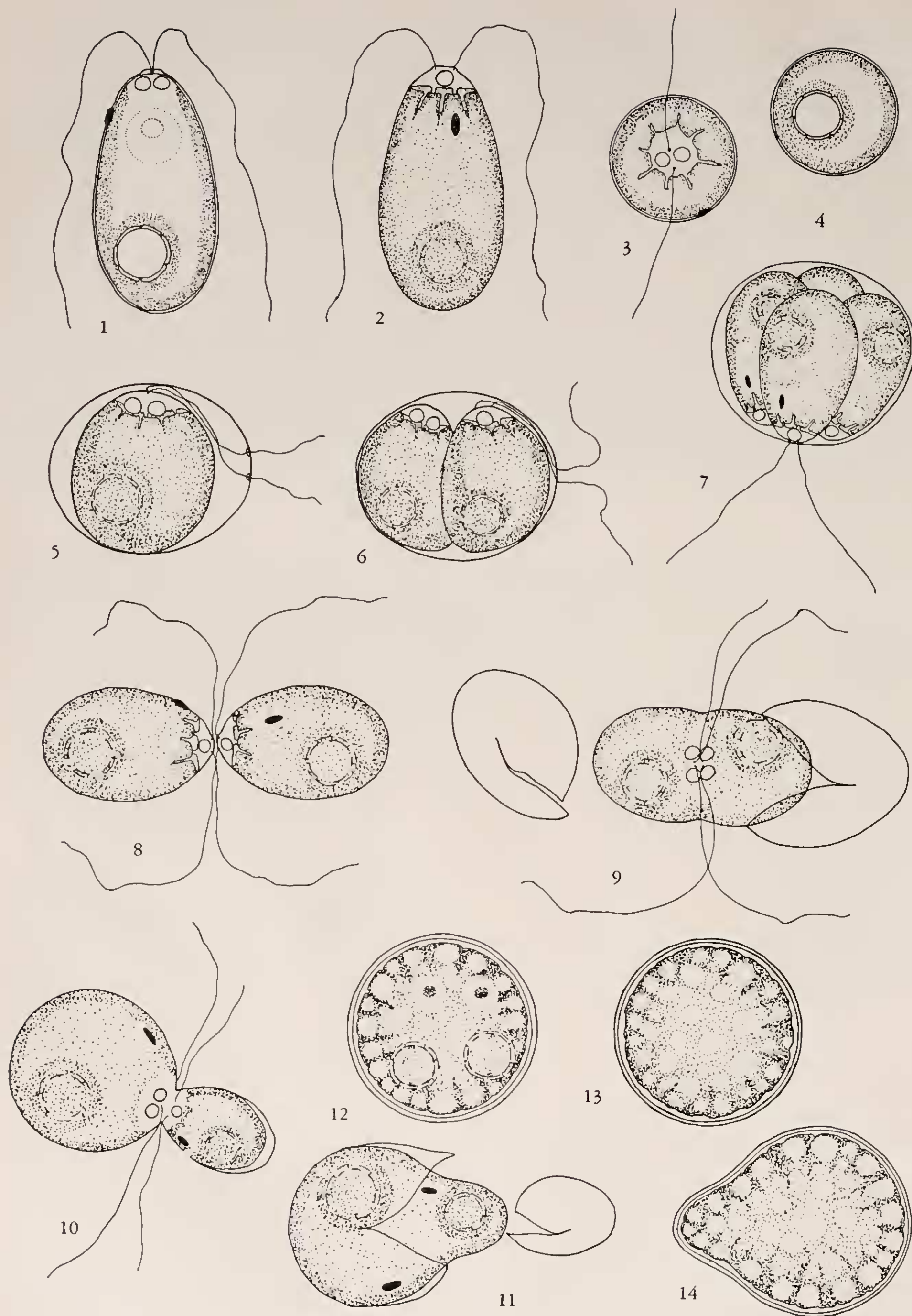
Table I

Growth of Chlamydomonas microhalophila after two weeks in Knop's medium with 5% soil-water supernatant and varying concentrations of Gulf sea water. Approximate salt content shown in parentheses

Concentration of sea water and salt content	Clone H-1	Clone H-2	Clone H-3	Clone H-4
4.3% (.36%)	++*	++	++	++
5.3% (.40%)	++	++	++	++
6.3% (.45%)	+	++	++	++
7.3% (.49%)	++	+	++	++
8.3% (.53%)	+	+	++	+
9.3% (.57%)	+	+	+	+
10.3% (.62%)	+−	+−	+−	+−
11.3% (.66%)	+−	+−	+−	+−
12.3% (.70%)	+−	+−	+−	+−
13.3% (.75%)	+−	+−	+−	+−
14.3% (.79%)	+−	+−	+−	+−
15.3% (.83%)	+−	+−	+−	+−
16.3% (.88%)	+−	+−	+−	+−
17.3% (.92%)	+−	+−	+−	+−
18.3% (.96%)	+−	+−	+−	+−
19.3% (1.00%)	+−	+−	+−	+−
20.3% (1.04%)	+−	−	−	+−
21.3% (1.09%)	+−	−	−	−
22.3% (1.13%)	−	−	−	−
23.3% (1.18%)	−	−	−	−

* Explanation of the symbols:

++ abundant growth (cultures dark-green),
\+ moderate growth,
+− scant growth,
− no growth apparent macroscopically.



FIGURES 1–14.

solution with various concentrations of Gulf sea water. Scant growth occurred in the solutions containing as much as 1.0% salts (Table I).

Hanging-drop preparations were used for observing living cells. All cultures were kept under constant fluorescent illumination at an intensity of 600 to 800 foot-candles and at a temperature of 15–17° C. Flagella were observed by staining motile cells with fixative described below, modified by increasing its iodine content to the point of saturation.

The following cytological methods were employed. Motile cells were taken from the surface of densely-populated stock cultures growing in liquid media and spread over the surface of sterile agar media in Petri dishes. These were illuminated at an intensity of 800 foot-candles for about six hours. The cultures were then observed, from time to time, under a stereoscopic binocular microscope for evidences of cell division. The maximum number of dividing cells and nuclear division figures was obtained at approximately 10 to 12 hours. Cells were fixed to glass slides and these were placed in Coplin jars containing fixative, as described by Buffaloe (1958). The writer also used the fixative which Cave and Pocock (1951) had modified from Johansen (1940), except that he reduced the iodine from 5 to 2.5 grams. The stained chromosomes were seen better when the starch granules were not as heavily stained, as in the case when a more concentrated iodine fixative is used. Slides were allowed to remain in the fixative for approximately three hours, and then were drained of excess fluid. The preparations were then flooded with aceto-carmine, prepared according to the method of Cave and Pocock (1951), and placed upon a hot plate with the thermostat set at 300° F. In a very short time vapors arose from the stain. After 1½–2 minutes steaming, during which the stain turned a deep red color, the slides were removed from the hot plate, drained and destained in 45% acetic acid for approximately 10 seconds. The slides next were placed in a mixture of equal volumes of 45% acetic acid and 95% alcohol for two minutes, and, then, 95% alcohol for 5 minutes. Finally, after the alcohol bath, a drop of Euparal was placed upon the area occupied by the fixed cells and covered with a cover glass.

All figures were drawn with the aid of a Spencer camera lucida, and reduced ½ in reproduction. The magnifications are: for Figures 1–4 and 15–18, 2000 ×; for Figures 5–9 and 19–24, 1750 ×; for Figures 10–14, 1500 ×. All figures were drawn from living material except Figure 12, which was stained with I_2-KI solution, and Figures 19–24 which were stained with aceto-carmine.

FIGURES 1–14. *Chlamydomonas microhalophila.* FIGURE 1, vegetative cell in median longitudinal optical section, showing nucleus, pyrenoid, stigma and form of chloroplast. FIGURE 2, vegetative cell in surface view. FIGURE 3, vegetative cell in anterior polar view showing the incised apex of the chloroplast and the relationship of the contractile vacuoles to the plane of attachment of the flagella. FIGURE 4, vegetative cell in transverse optical section at the level of the pyrenoid. FIGURES 5–7, asexual reproduction; in this case, parental flagella functional during division. FIGURE 5, note 90° rotation, protoplast rotated 90° from longitudinal axis of cell wall; cell is still motile due to incomplete withdrawal of flagella. FIGURE 6, first cleavage completed. FIGURE 7, second cleavage completed, with the four daughter cells rotated in line with the longitudinal axis of mother cell wall. FIGURES 8–18, sexual reproduction. FIGURE 8, gametes after initial entanglement of flagella which are now completely separated; gametes probably attached by a protoplasmic thread. FIGURE 9, fusion in progress; flagella still slightly motile; note discarded gamete walls. FIGURE 10, pseudoheterogamous pair. FIGURE 11, the same, somewhat later. FIGURE 12, zygote stained with I_2-KI, showing two distinct nuclei and pyrenoids 48 hours after plasmogamy. FIGURES 13, 14, dormant zygotes.

Observations

Morphology and reproduction

The organism is ellipsoidal. The anterior and posterior poles are broadly rounded, but the anterior one is more acuminate than the posterior. Cell size ranges from 8.5 μ–20 μ in length and 5 μ–12 μ in width with the population averaging 14 μ in length and 8.5 μ in width. Young cells from germinating zygospores may be as small as 6 μ in length and 3 μ in width. The variation in cell size is a reflection of phases of development after liberation of daughter cells from the parent cell walls. The papilla, which is most clearly visible in small, young cells, is truncate, and it becomes obscured with increase in cell size and wall thickness (Figs. 1 and 2). Two small flagellar orifices may be observed when the protoplast contracts from the wall during cell division (Fig. 5).

The chloroplast in median, optical section is a relatively thick-walled, hollow, ovoidal structure open at the anterior end. Here the chloroplast displays an irregularly scalloped margin (Figs. 2 and 3). The inner surface of the plastid is slightly undulate. The single spherical pyrenoid always lies in a lateral position, in a thickening of the chloroplast, in the posterior third of the cell (Fig. 1). The elliptical, disc-shaped stigma is embedded in the periphery of the anterior third of the chloroplast. The cell wall protrudes slightly at the region of the stigma (Fig. 1). The size and form of the stigma were constant in all cells observed.

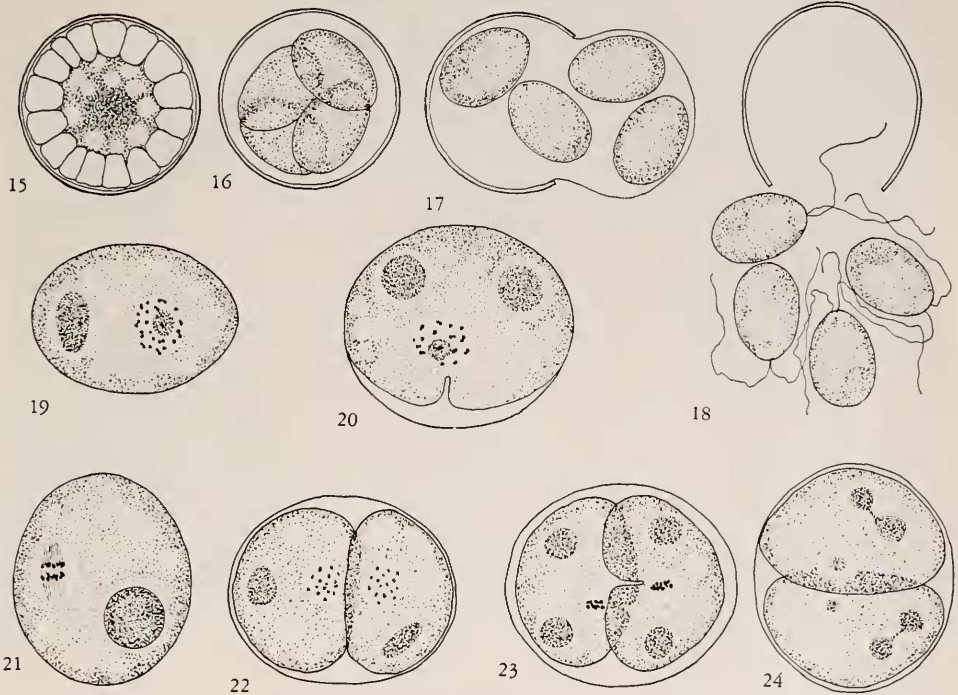
The nucleus is anterior in the colorless cytoplasm. Both in living and stained cells a large nucleolus is visible, but a centrosome could not be demonstrated even with Heidenhain's iron haematoxylin, as reported for *C. terricola*. Two contractile vacuoles occupy the anterior portion of the protoplast and always lie in a plane perpendicular to that of the attachment of the flagella (Figs. 3 and 25). The latter are considerably longer than the cell. Flagella nearly twice the cell length are very common among the smaller cells.

The presence of zygotes in the twenty clonal cultures proved the organism is homothallic. The gametes are not distinguishable from the vegetative cells, except for their smaller size, an indication that, as in most species of *Chlamydomonas,* only young cells are sexually active. The gametes are isogamous; although fusion between gametes of unlike size was observed, this is explained by the fact that they are in various stages of maturation (Figs. 10 and 11).

In sexual union two gametes, with free flagella, repeatedly and vigorously approach each other in the region of the papillae as observed by Bold (1949) in *C. chlamydogama.* After one to two hours of this behavior, they become almost motionless. During this process the pairs do not move very far from a given point, another similarity to *C. chlamydogama.* A protoplasmic thread, as described by Bold (1949) and Lewin and Meinhart (1953), probably unites the two cells at this stage, but because of the constant movement of the cells this thread was not observed with certainty. The space between the papillae and four free-moving flagella is very good evidence that this thread does exist in this species (Fig. 8).

After two to three hours, a permanent union of the gametes occurs at the region of the papillae, as the flagella continue to beat very feebly. The gamete walls are shed (Fig. 9) as in *C. chlamydogama.* The union of the gamete protoplasts is very slow, sometimes covering a period of 5 to 6 hours. The flagella disappear

and within 24 hours a zygote is formed. If the gametes are of equal size a spherical zygote results (Fig. 13); if the gametes are of unequal size, a "pear-shaped" zygote is formed, resulting possibly from the denser consistency of the larger cell (Fig. 14). A thick wall is secreted around the zygote. Both pyrenoids and nuclei are visible in the zygotes for as long as 48 hours (Fig. 12). In some

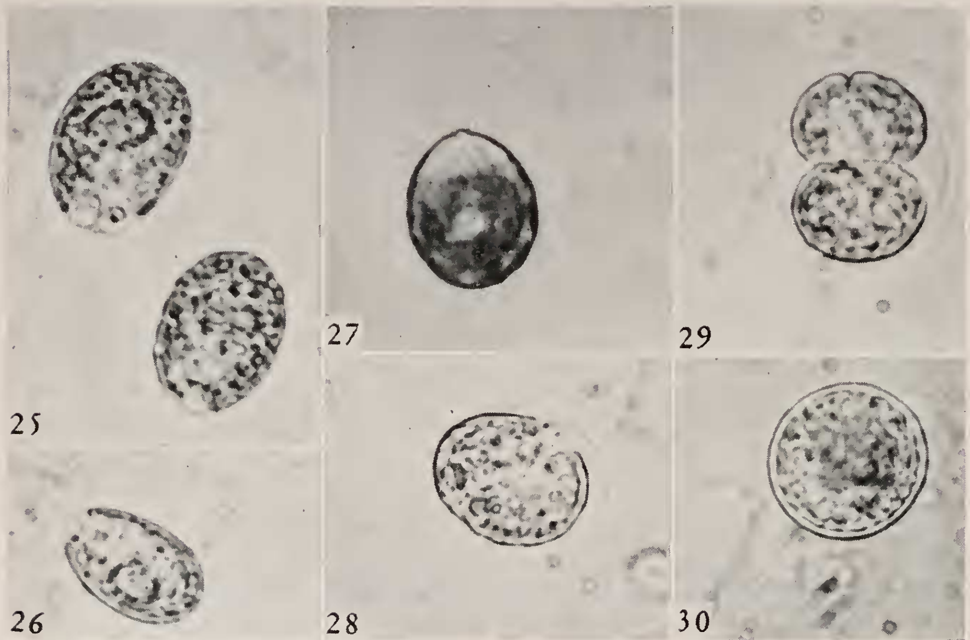


FIGURES 15–24. *Chlamydomonas microhalophila.* FIGURE 15, six-month-old zygote in median optical section with large oil droplets concentrated at the periphery. FIGURES 16–18, zygote germination. FIGURE 19, early prophase of nuclear division showing some of the chromatin bodies before condensation into chromosomes, pyrenoid elongating. FIGURE 20, pyrenoid has divided and cytokinesis is being initiated in the region of the nucleus; the latter in late prophase showing 16 chromosomes. FIGURE 21, anaphase stage with approximately 16 chromosomes moving toward the opposite poles; the pyrenoid has not divided nor has cytokinesis been initiated. FIGURE 22, first cleavage is completed, and nuclei are in prophase stage for second division; pyrenoid in one of the cells is elongating. FIGURE 23, second cleavage, pyrenoids divided, and chromosomes at metaphase. FIGURE 24, division of pyrenoids preceding second cleavage.

instances, the pyrenoids were visible after several weeks. The zygote enlarges to as much as 22 μ in diameter as it matures. Dormant zygotes several weeks old accumulate droplets of colorless oil in the periphery, with the chlorophyll concentrated in the center (Figs. 15 and 30). With increasing age the oil droplets enlarge. Very large, reddish-orange colored oil droplets, as confirmed by Sudan III, were observed in dormant zygotes 6 months old.

To effect germination, zygotes which had dried on agar, under illumination

of 800 foot-candles for a period of over six months, were flooded with distilled water and kept in darkness for three days. After this, a small volume of soil-water supernatant was added and the tubes illuminated. The first germination occurred within 48 hours. At the end of six days most of the zygotes had liberated four small, motile daughter cells approximately 6 μ in length (Figs. 16, 17 and 18).



FIGURES 25–30. *Chlamydomonas microhalophila.* Photomicrographs of living cells, except Figure 27. FIGURE 25, mature cell just prior to division; note contractile vacuoles. FIGURE 26, immature cell, median optical section; note unilateral pyrenoid and chromatophore thickness. FIGURE 27, cell treated with I_2-KI, showing flagella. FIGURE 28, rotation of protoplast prior to cleavage. FIGURE 29, beginning of second cleavage. FIGURE 30, maturing zygote.

Cytology

As in many species of *Chlamydomonas*, the protoplast rotates 90° within the wall prior to cell division. Cytokinesis in this species is initiated by the appearance of a furrow in the region of the nucleus, and opposite the position of the pyrenoids or dividing pyrenoid (Fig. 20). The first division occurs perpendicular to the longitudinal axis of the cell. This coincides with the description given by Kater (1929) for *C. nasuta,* Akins (1941) for *Carteria crucifera,* Bold (1949) for *C. chlamydogama,* and by Buffaloe (1958) for four species of *Chlamydomonas.*

The two daughter cells usually undergo one more division which takes place in the same manner (Figs. 23 and 29). Cytokinesis is unilateral in the cytoplasm surrounding the nucleus (Figs. 20 and 23). Buffaloe (1958) reported that for four species of *Chlamydomonas* he studied, there was no exact synchrony between the division of the nucleus and the division of the pyrenoid. The same is true for the present organism, but there is synchrony between division of the pyrenoid and

cytokinesis. It was observed many times that cytokinesis is initiated while the nucleus is still in the prophase stage (Fig. 20). The division of the pyrenoid appears always to signal the inception of cytokinesis. The division of the pyrenoid follows its elongation (Figs. 19 and 24). Nuclear division may begin before or after the initiation of cytokinesis (Figs. 20 and 21).

Each dividing mother cell usually gives rise to two or four daughter cells (Figs. 7, 22 and 23). Sometimes 8 and in exceptional cases 16 and even 32 cells were observed. Cell division generally takes place in non-motile cells, the flagella of which have been withdrawn. Occasionally, the mother cell does not become entirely non-motile, and following one or two successive bipartitions, two or four daughter cells propelled by the original flagella may be observed swimming about slowly and in cumbersome fashion (Figs. 5, 6 and 7).

The interphase nucleus is approximately 4 μ in diameter and possesses one large nucleolus. During early prophase 30 or more irregularly shaped, darkly-stained bodies may be observed, scattered about the nucleolus (Fig. 19). In later prophases, the nucleolus disappears and the darkly-stained chromosomes number approximately 16 (Fig. 22). These 16 spherical and slightly oblong bodies were never observed to form a ring in the metaphase as reported by Buffaloe (1958) for *C. reinhardti.* The polar view of the metaphase stage appears rather as a solid disc consisting of irregularly scattered chromosomes. Fewer than 16 ± 1 chromosomes were never observed. Early anaphase stages with spindle fibers clearly visible also exhibited two sets of approximately 16 chromosomes moving toward opposite poles (Fig. 21).

Discussion

On the basis of the morphological and cytological data reported in this paper, the writer attempted to ascertain the specific identity of the *Chlamydomonas* studied by consulting the literature. The organism is somewhat suggestive of *C. terricola* Gerloff (1940) but differs from it clearly in a number of respects such as position of the stigma, nuclear position, chromosome number, behavior of gamete walls at copulation and nature of the zygote wall, among others. It differs from *C. intermedia* Klebs in the anterior position of the nucleus and posterior position of the pyrenoid. Further search of the literature has failed to reveal an organism with a combination of attributes like those of the organism studied in the investigation here reported. Therefore, it is described as a new taxon, *C. microhalophila* sp. nov., the specific name an allusion to its tolerance of a relatively high concentration of salt, as compared to other species. The specific diagnosis follows:

Chlamydomonas microhalophila [3]

Cellulae ellipsoideae, ad polum anteriorem paululum attenuatae; magnitudo cellularum, secundum aetatem, 8.5–20 μ long. atque 5–12 μ lat. Chromatophorus cavus urceolatus, paululum infra polum anteriorem abrupte terminatus atque incisus, incrassatione unilaterali prope basim, pyrenoideum prominens continente, praeditus. Stigma anterius protuberans; nucleus anterior. Duae vacuolae pulsantes atque duo flagella longitudine corpori aequa aut longiora. Numerus

[3] The writer is grateful to Dr. Hannah T. Croasdale for preparing the Latin diagnosis.

chromatosomatum (n) = 16. Planta homothallica, in reproductione sexuali isogamica, membranis gametarum tempore coniunctionis sexualis, zygotum efferentis, omnino adiectis. Zygota matura usque ad 22 μ diam., membranan levem habentia, in quattuor cellulas filias plerumque germinantia.

Origo: In doliis magnis aquae plenis in loco Supply Department dock, M.B.L., Woods Hole, Mass. dicto.

Summary

1. Morphological and cytological observations of a microhalophilic alga are described and illustrated.

2. Clonal cultures isolated from a barrel of water at the Marine Biological Laboratory, Woods Hole, Massachusetts, proved to be members of an undescribed species of *Chlamydomonas*.

3. The organism is described as *C. microhalophila* sp. nov., a member of the *Chlamydella* section of the genus.

4. The organism tolerates concentrations of salts (predominantly NaCl) up to approximately 1.0%, and responds by marked increase in growth in concentrations up to approximately 0.5%.

5. Its homothallic sexual reproduction and zygote germination are described and figured.

6. Its chromosome number has been determined as $n = 16 \pm 1$.

7. Cultures of the organism have been deposited in the Culture Collection of Algae, Department of Botany, Indiana University, and herbarium specimens have been sent to the Chicago Natural History Museum.

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